REMARKS

The present application relates to inbred maize line PH951. Claims 1-36 are pending in the present application. Claims 7, 9, 16 and 25-28 have been amended. No new matter has been added by way of amendment. Applicant respectfully requests consideration of the claims in view of the following remarks.

Detailed Action

Applicant acknowledges that because this application is eligible for continued examination under 37 C.F.R. § 1.114 and the fee set forth in 37 C.F.R. § 1.17(e) has been timely paid, the finality of the previous Office Action has been withdrawn pursuant to 37 C.F.R. § 1.114. Applicant further acknowledges that Applicant's submission filed on October 13, 2005 has been entered.

Claim Objections

The Examiner objects to claim 16 and suggests "wherein seed is allowed to form" be replaced with --and harvesting seed-- for clarification. Applicant has amended the claim as suggested by the Examiner, thus alleviating this objection.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 13-14, 25-30 and 34 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner states claim 13 is indefinite "for omitting essential steps." *See* Office Action, pp. 2-3.

Applicant traverses this rejection. Applicant has included "repeating steps (c) and (d) to produce backcross progeny plants that comprise the desired trait and comprise at least 95% of the alleles of inbred line PH951 at the SSR loci listed in Table 4" in claim 13. Applicant further asserts the use of molecular marker profiles by those of ordinary skill in the art in backcrossing is also clearly supported by the scientific literature. For example, see Ragot, M. *et al.* (1995) Marker-assisted backcrossing: a practical example, in *Techniques et Utilisations des Marqueurs Moleculaires (Les Colloques*, Vol. 72, pp. 45-56 (attached as Appendix 1), and Openshaw *et al.*, (1994) Marker-assisted Selection in Backcross Breeding, Analysis of Molecular Marker Data,

pp. 41-43 (attached as Appendix 2). Specifically, Ragot *et al.* states in the first sentence of the summary "[t]hat molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed," and, in the first sentence of the introduction, "[b]ackcrossing has been a common breeding practice for as long as elite germplasm has been available." Therefore, Applicant has claimed in the manner used by those of ordinary skill in the art to characterize backcross conversions.

Claim 14 is rejected as rejected as indefinite as depending from rejected claim 13.

Applicant traverses this rejection for the reasons asserted *supra*. Claim 14 is definite and does include the essential method steps of claim 13.

Regarding claims 25 and 27-30, the Examiner states that the claims "do not incorporate all elements of the parent claim 15," specifically that the "plant of parent claim 15 does not contain a single locus conversion, a dominant or recessive allele/transgene." *See* Office Action, p. 3.

Applicant respectfully traverses. Claim 15 specifically claims a maize plant having all the physiological and morphological characteristics of inbred line PH951. Claim 15 encompasses maize plants having the characteristics of inbred line PH951. Claims 25 and 27-30 claim the maize plant of claim 15 with these additional limitations, which are not necessarily present in the maize plant of claim 15. The presence of these additional limitations does not mean that claims 25 and 27-30 do not possess all limitations of claim 15; these claims still require a maize plant having the physiological and morphological characteristics of inbred line PH951. Because claims 25 and 27-30 do incorporate all elements of claim 15, they are in accordance with the requirements of § 112, second paragraph.

The Examiner further states that claims 28-30 are indefinite in the "recitation of 'male sterility' because the plant of parent claim 15, PH951, is male fertile." *See* Office Action, p. 3.

Applicant respectfully traverses. It would be understood by one of ordinary skill in the art that the deposited line can be manipulated and made male sterile by methods such as backcrossing, as described in the specification. *See, e.g.*, specification, pp. 2-4. "It should be understood that the inbred can, through routine manipulation by detasseling, cytoplasmic genes, nuclear genes, or other factors, be produced in a male-sterile form." *See* specification, p. 36, ll. 20-22. One of skill in the art also understands that transgenes can be incorporated into the inbred line in a similar manner. *See* specification, pp. 38-48. Male sterile conversions have been made

to inbred lines since the 1950's, and transgenic conversions have been made to inbred lines since the early 1990's. Both are routinely made, and the language and meaning of these claims are well understood by plant breeders. The primary purpose of the requirement of definiteness of claim language is to "ensure that the scope of the claim is clear so the public is informed of the boundaries." MPEP § 2173. That objective has been satisfied by claims 28-30.

Claim 34 is rejected as indefinite in the recitation of "using" without any active method steps. *See* Office Action, p. 3.

Applicant traverses this rejection. The specification states "[p]lant breeding techniques known in the art and *used* in a maize plant breeding program include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, restriction fragment length polymorphism enhanced selection, genetic marker enhanced selection, making double haploids, and transformation. Often a combination of these techniques are used." Specification, p. 4, ll. 8-13 (emphasis added). Therefore, Applicant asserts that one of skill in the art would know the meaning of the term "using" in claim 34.

In light of the above amendments and remarks, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-12 remain rejected and claims 17-21, 23, 25-28, 31-32 and 34-36 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The Examiner asserts that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Examiner states the rejection is repeated for claims 1-12 and applied to new claims 17-21, 23, 25-28, 31-32 and 34-36 for the reasons of record set forth in the Office Action of July 13, 2005. *See* Office Action, p. 3.

Applicant respectfully traverses this rejection. Applicant reiterates that the written description requirement of § 112, first paragraph has been fulfilled by depositing seeds of PH951 in a public depository and by referencing the deposit in the specification. *See* specification, p. 65, ll. 2-28; *see also Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 965, 63 U.S.P.Q.2d 1609, 1613 (Fed. Cir. 2002) (stating that the written description requirement of § 112, ¶ 1 may be

fulfilled by depositing material in a public depository, where the deposited material is not accessible in writing, and where reference to the deposit is made in the specification). This deposit not only describes inbred maize line PH951 but also the hybrid maize plants, plant parts, and seeds grown of claims 1-12, 17-21, 23, 25-28, 31-32 and 34-36. In a prior case before the Board of Patent Appeals and Interferences, the Board determined that where claims to an inbred maize plant satisfied the written description requirement, claims to the F1 hybrid seed and plants with the inbred maize plant as a parent also satisfied the written description requirement. *See Ex parte Carlson* (B.P.A.I. 2005). The Board therein stated:

> All that is required by the claims is that the hybrid has one parent that is a plant of corn variety [inbred]. Since the examiner has indicated that the seed and the plant of the corn variety [inbred] are allowable . . . there can be no doubt that the specification provides and adequate written description of this corn variety. In addition, the examiner appears to recognize (Answer, page 25) that appellant's specification describes an exemplary hybrid wherein one parent was a plant of the corn variety [inbred]. . . Accordingly, it is unclear to this merits panel what additional description is necessary.

Ex parte Carlson, p. 16. Here, Applicant has done just what the applicant in *Ex parte Carlson* did, that is claim hybrids having one parent that is a plant of an inbred variety. Further, Applicant reiterates that the specification contains an example of a hybrid produced by PH951 in the application as filed. *See* specification, pp. 57-58, Table 3. Thus, under *Ex parte Carlson*, "it is unclear . . . what additional description is necessary." *See Ex parte Carlson*, p. 16; *see also Regents of Univ. of Cal.*, 119 F.3d at 1569, 43 U.S.P.Q.2d at 1406 (stating that an Applicant is "not required to disclose every species encompassed by their claims even in an unpredictable art"). Additionally, claim 15, directed towards a plant having all the morphological and physiological traits of PH951 wherein PH951 was deposited with the ATCC, is only rejected on obviousness-type double patenting grounds, which, as described *infra*, has been obviated by filing a terminal disclaimer with this amendment.

Applicant reiterates that each member of the genus of hybrids which has PH951 has a parent and which is encompassed by claims 1-12, 17-21, 23, 25-28, 31-32 and 34-36 shares the identifying structural feature of the cells and/or chromosomes of inbred line PH951. An Applicant's claims are described where they set forth and define "structural features commonly possessed by members of the genus that distinguish them from others." *Regents of Univ. of Cal. v. Eli Lilly & Co.*, 119 F.3d 1559, 1568, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997) (emphasis

added). One of skill in the art, utilizing technology well known in the art, could identify any member of the claimed genus.

The Examiner further states that the specification "does not describe the functions (i.e., morphological and physiological traits) of the claimed hybrids, and does not correlate the functions of the hybrids with the structure of the genetic complement or set of chromosomes from PH951" and that "the claimed hybrids do not have the entire genomic characteristics of PH951, but only one set of chromosomes of PH951," and therefore the Examiner states that "even if one assumes that the SSR profile is a proper way to describe a hybrid, then it will require the SSR profiles of both parents to identify the hybrid not just the SSR profile of one of the parents." *See* Office Action, p. 6.

Applicant respectfully traverses this rejection. Most importantly, Applicant points out that the SSR profile of PH951 is sufficient to describe the claimed hybrids, and the Examiner's assertion that to describe the claimed hybrids using SSR requires "the SSR profiles of both parents" is improper. It is vital to conceptually understand that all F1 hybrid seed produced with PH951 will inherit the stable genetics of PH951. Therefore, knowing the SSR profile of PH951 permits the identification of any F1 hybrid produced with PH951 as one parent, as every such hybrid will have at least one set of PH951 chromosomes, and is therefore able to be identified using the SSR profile of PH951. Applicant has further described the SSR marker profile in Table 4 of the specification. *See* specification, Table 4, pp. 61-64. Given this information, one of ordinary skill in the art could identify any F1 hybrid with PH951 as a parent. Thus, Applicant respectfully submits the claimed invention is in accordance with the written description guidelines.

The Examiner states "where the breeding involves unknown various non-PH951 parents, all F1 hybrids will not receive the same set of chromosomes from each of the parents involved in the breeding." *See* Office Action, p. 7.

Applicant reiterates that each F1 hybrid which has PH951 as a parent and which is encompassed by claims 1-12, 17-21, 23, and 25-28 contain at least one set of chromosomes of inbred line PH951. Thus, these claims set forth "structural features commonly possessed by members of the genus that distinguish them from others," as only F1 hybrids with PH951 as a parent would have a complete set of PH951 chromosomes. *Regents of Univ. of Cal.*, 119 F.3d at 1568, 43 U.S.P.Q.2d at 1406. The claimed F1 hybrids are therefore described in such a way that

distinguishes them from other hybrids, which is sufficient to meet the written description requirement. *See id.*

Further, Applicant has not stated that all F1 hybrids made with PH951 would be phenotypically the same. It is true that genetics correlate with phenotype, and that the more highly related two individuals are genetically, the more similar their phenotype is likely to be. It is also true that if one desired to produce an F1 hybrid with the characteristics of the F1 hybrids disclosed in Table 1 and Table 3, one of skill in the art would prefer to utilize PH951 rather than spending the time and resources to develop a novel inbred. However, the written description requirement does not mandate a description by phenotype. At its foundation, the written description requirement serves an evidentiary function of making certain that the Applicant is in possession of a specific characteristic that identifies their claimed invention. The molecular marker data provided by Applicant in Table 4 serves this purpose. *See* specification, Table 4, pp. 61-64. The other inbred is not the point of patentability, nor is it what is being claimed. Rather, the relevant claims are drawn precisely to what is described, an F1 hybrid with the identifiable and unique molecular profile of PH951.

The Examiner states that new claims 13-14, 21, and 23 are rejected because "the SSR loci listed in Table 4 are not structurally described", and that "step (e) of claim 11 fails to describe the number of times steps (c) and (d) have to be repeated to produce backcross progeny plants with the desired trait and essentially all the morphological and physiological characteristics of the inbred." *See* Office Action, p. 9.

As an initial matter, it appears that the Examiner inadvertently referenced the incorrect claim number in the Office Action. The method claim containing the steps referred to is claim 13, not claim 11. Assuming this, Applicant respectfully traverses this rejection. Primers for the SSR markers listed in Table 4 are publicly available as stated in the present application. Applicant respectfully directs the Examiner's attention to page 60, lines 19-23 of the specification where it states that "[p]rimers used for the SSRs reported herein are publicly available and may be found in the Maize GDB using the World Wide Web prefix followed by maizegdb.org (maintained by the USDA Agricultural Research Service), in Sharopova *et al.* (Plant Mol. Biol. 48(5-6):463-481), Lee et al (Plant Mol. Biol. 48(5-6); 453-461), or reported herein. Some marker information may be available from Paragen." A printout from the maize GDB website using bnlg1014 as an example has been included with this response as Appendix 3.

The printout shows the extent of amount of marker information available on the maize GDB, which includes primer sequences and map information. As explained in the specification, primer sequences for the public SSR markers listed in Table 4 can be easily obtained through the world wide web. *See* specification, p. 60, ll. 19-21 (describing the Maize GDB).

Further, Applicant asserts that the alleles of inbred line PH951 disclosed in the SSR profile of Table 4 is an identifying physical characteristic that describes the genus of minor variance of inbred line PH951. The SSR profile of PH951 is disclosed for numerous markers distributed throughout the genome as indicated by the Bin number of the marker, which denotes the marker location. A plant comprising 95% of the alleles of PH951 as disclosed in Table 4 would be produced, for example, by repeated backcrossing to PH951. A backcross conversion of PH951 as claimed in the instant application is described as comprising 95% of the alleles disclosed in Table 4. *See* specification, Table 4, pp. 61-64.

It is undisputed that fingerprinting with molecular markers is widely used for characterizing germplasm. Specifically, SSR profiles are known and can be practiced by one of ordinary skill in the art in maize breeding. One of ordinary skill has been enabled by the deposit to make and use minor variants of inbred corn line PH951, and one of ordinary skill in the art uses SSR markers to characterize backcross conversions of an inbred. Applicant has claimed in the manner used by those of ordinary skill in the art to characterize backcross conversions.

Regarding the failure of step (e) to describe the number of times steps (c) and (d) are repeated, Applicant refers to the response to Examiner's similar rejection under 35 U.S.C. § 112, second paragraph, *supra*. For similar reasons, step (e) of these claims is adequately described.

The Examiner states that new claims 25-27 are rejected because "the claims do not place any limitation on the traits conferred or affected by the single locus conversion," and that the claims "broadly encompass single loci that have not been discovered or isolated." *See* Office Action, p. 10. The Examiner also states that claims 28-30 are included in the rejection because the specification "provides no description of any plant produced by classical breeding methods such as backcrossing or recurrent selection," that no "individual genes conferring the desired traits have been characterized," and the relevant genes as claimed have not been isolated. *See* Office Action, p. 10.

Applicant respectfully traverses this rejection. The relevant claimed subject matter in claims 25-27 is the plant of claim 15 comprising a transgene or gene conversion. The

specification teaches multiple ways of introgressing or transforming a maize plant with various genes which confer advantageous traits desired in the plant. *See* specification, pp. 38-40. The specification also teaches many transgenes that could be inserted into the plant of claim 15. *See* specification, pp. 40-48. Applicant further notes that the claims are specifically drawn to a single gene conversion, and that phenotypes resulting from multigenic interactions are not the subject matter of these claims. For example, numerous exemplary transgenes for improved nutritional quality are taught on page 47 of the specification. There are many examples of single gene conversions which affect nutritional quality, see for example, as taught in the specification transforming a plant with an antisense gene of stearoyl-ACP desaturase to increase stearic acid content of the plant, *see* page 47, ll. 4-7, introduction of a phytase-encoding gene that would enhance breakdown of phytate, adding more free phosphate to the transformed plant, *see* page 47, ll. 9-12. In addition, see U.S. Patent No. 5,936,145, issued August 10, 1999, which is prior to the filing date of the instant application. Claim 39 reads as follows: "[t]he single gene conversion of the corn plant of claim 29, where the gene confers enhanced yield stability." Thus, a single gene that confers enhanced yield stability was known in the art prior to the filing date of the instant application. One of skill in the art would recognize that it is common to transform a maize plant with various genes in order to confer desired traits to the maize plant.

The Examiner further states that claims 31-32 are included in the rejection "because the claims read on a method for crossing PH951 with a multitude of non-exemplified breeding partners which have not been characterized either morphologically or genetically." *See* Office Action, p. 10. Claims 34-36 are likewise rejected "because the claims require the use of a multitude of non-exemplified molecular markers." *See* Office Action, p. 11.

Applicant respectfully traverses this rejection. Claims 31-32 and 34-36 are directed towards methods for producing a maize plant derived from PH951 and developing a maize plant in a plant breeding program where the maize plant of claim 15 is used as a source of breeding material. The language of claims 31-32 and 34-36 makes clear that the maize plant of claim 15 must be used as breeding material in the breeding program described by claims 31-32 and 34-36.

Plant breeding techniques are well known to individuals skilled in the art. The specification describes many of these known techniques. *See* specification, p. 1, l. 18-p. 8, l. 2. In particular, the specification discusses the role of an inbred maize line in a plant breeding program:

> Plant breeding techniques known in the art and used in a maize plant breeding program include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, restriction fragment length polymorphism enhanced selection, genetic marker enhanced selection, making double haploids, and transformation. Often a combination of these techniques are used. The development of maize hybrids in a maize plant breeding program requires, in general, the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Maize plant breeding programs combine the genetic backgrounds from two or more inbred lines or various other germplasm sources into breeding populations from which new inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which of those have commercial potential.

Specification, p. 4, ll. 8-21.

As the specification makes clear, one of ordinary skill in the art would know how a maize inbred line is to be used in a plant breeding program. As taught by the specification, the maize inbred is used as a source of germplasm in creating new hybrid lines. It is thus clear from the specification, and to one of ordinary skill in the art, how PH951 would be employed in a plant breeding program.

One skilled in the art would thus recognize that Applicant was in possession of the invention described in claims 1-12, 17-21, 23, 25-28, 31-32 and 34-36 as of the filing date of the application. Accordingly, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph.

Double Patenting

The Examiner rejects claims 15-16 and 22 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 2 and 24 of U.S. Patent No. 6,756,530. The Examiner states that although the conflicting claims are identical, they are not patentably distinct from each other because the claims in both application and the patent are directed to maize plants having all the morphological and physiological characteristics of inbred maize line PH951 and parts of said plants. *See* Office Action, p. 11-12.

Applicant is herein submitting a Terminal Disclaimer in compliance with 37 C.F.R. § 1.321(c), which disclaims any term of a patent issuing from this application which would extend beyond the term of co-pending U.S. Patent No. 6,756,530. Therefore, Applicant submits that the

claims are in proper form for allowance and respectfully requests reconsideration and withdrawal of the obviousness-type double patenting rejection.

Conclusion

In conclusion, Applicant submits in light of the above amendments and remarks, the claims as amended are in a condition for allowance, and reconsideration is respectfully requested. If it is felt that it would aid in prosecution, the Examiner is invited to contact the undersigned at the number indicated to discuss any outstanding issues.

Please charge Deposit Account No. 26-0084 the amount of $130.00 for the enclosed Terminal Disclaimer and $120.00 for a one month extension of time from March 19, 2006 to April 19, 2006, under the provision of 37 C.F.R. § 1.136(a). No other fees or extensions of time are believed to be due in connection with this amendment; however, consider this a request for any extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Reconsideration and allowance is respectfully requested.

Respectfully submitted,

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Marker-assisted backcrossing: a practical example

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Summary

That molecular markers allow fast recovery of recurrent parent genotype in backcross programs is undisputed. Restriction Fragment Length Polymorphisms (RFLP's) were used in maize to introgress by backcross a transgene construct, containing phosphinothricin resistance and insecticidal protein genes, from a transformed parent into an elite inbred line. At each generation plants carrying the transgene construct were selected based on their phosphinothricin resistance, and further characterized with RFLP's. Both maximum recovery of recurrent parent genotype and minimum linkage drag were taken into account for marker-based selection. Embryo rescue was used to shorten generation time. Progress towards recurrent parent genotype was spectacular. Levels of recurrent parent genotype recovery which would normally be observed, in the absence of selection, in the BC_6 generation were obtained at the BC_3 generation, about one year after BC_1 seeds had been planted. Besides the evidence already provided by RFLP's, phenotypic evaluation of the backcross-derived near-isogenic lines will constitute an additional check of the completeness of the conversion.

Introduction

Backcrossing has been a common breeding practice for as long as elite germplasm has been available. It has mainly been used to introgress single Mendelian traits, such as disease resistances or quality factors, into elite germplasm (Allard 1960; Hallauer and Miranda 1981). One of the most attractive attributes of backcrossing is that it allows to perform targeted modifications without disrupting the existing overall genetic balance of the recurrent parent.

However, production of fully converted near isogenic lines through classical backcrossing procedures is a lengthy procedure, if at all possible. Theoretically, a minimum

APPENDIX 1

of seven classical backcross generations are required to recover more than 99% of recurrent parent genotype, assuming no linkage drag. The attractiveness of classical backcross procedures is therefore substantially diminished for crops, such as maize (*Zea mays* L.), where the turn-over of elite cultivars is very fast. In addition, full recovery of recurrent parent genotype is usually not achieved through classical backcrossing, which may result in deleterious agronomic effects. Murray *et al.* (1988) reported about 90% recurrent parent genotype recovery in two BC_{10}-equivalent conversions (A632Ht and A632Rp) of the maize line A632. The conversions had retained respectively 4 and 7 donor fragments in addition to the one carrying the gene of interest.

Reduction in the number of backcross generations needed to obtain fully converted individuals has been shown theoretically, or from simulations, to be achievable through the use of molecular markers (Tanksley *et al.* 1989; Hospital *et al.* 1992; Jarboe *et al.* 1994). Because they provide thorough characterization of the genetic variability at each backcross generation, markers allow to take full advantage of this variability by applying the highest possible selection intensity.

Efficiency of marker-assisted backcrossing was investigated through an experiment aimed at introgressing a single genetic factor (a transgene construct) from a donor into a recipient maize line.

Materials and methods

Plant Material

A hemizygous transgenic maize line of Lancaster origin was used as donor parent to introgress its transgene construct, through repeated backcrossing, into a recipient parent from the Stiff Stalk germplasm group. Both parents are proprietary elite lines. The transgene construct carries both a phosphinothricin resistance gene and synthetic genes encoding the entomotoxic fragment of the CryIA(b) *Bacillus thuringiensis* protein (Koziel *et al.* 1993). Transformation was achieved through microprojectile bombardment (Koziel *et al.* 1993) and resulted in a single insertion (*Bt* locus), on chromosome 1 (Figure 1).

Backcross protocol

The F1 progeny of the cross between the donor and the recipient was screened for the presence of the transgene construct by applying Basta, a phosphinothricin-based herbicide, onto each plant. Resistant individuals were then used to generate BC_1 progeny.

For each backcross generation, except the BC_4, individuals were planted in multipots and sprayed with Basta to eliminate those which did not carry the transgene construct. To avoid the stress resulting from treatment with Basta, BC_4 plants carrying the transgene construct were identified using Southern blots probed with the *pat* and *Bt* genes. Resistant plants were transplanted in an open-soil greenhouse and leaf-sampled for molecular marker analyses. Results of marker an... flowering. A single plant was... rescued and transferred onto t... embryos first underwent a gre... culture medium, before being... average, four months.

Molecular marker analys...

Restriction Fragment Le... genotypes in all four genera... chemiluminescent techniques. ... were chosen from among those... provided coverage of the entir... contained two loci tightly linke... recombination units away (Figu... BC_{n+1} generation comprised bo... or tightly linked ones, and addi... selected BC_n plant was heteroz... independent reference populati... generation.

Selection procedure

At each generation plants... recurrent-parent-genotype and ... attempt to integrate both crite... missing values were not include... contributed to the selection proc... best ranking one of those for w... for the BC_3 selection) was avail...

Results and discussion

Selection for the gene o...

The observed segregatio... significantly different ($P < 0.05$...

Recurrent parent genoty...

Statistics for the genoty... performed taking the whole g... backcross-derived plant therei...

recover more than 99% of recurrent
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ckcrossing, into a recipient parent
are proprietary elite lines. The
sistance gene and synthetic genes
llus thuringiensis protein (Koziel *et*
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chromosome 1 (Figure 1).

the recipient was screened for the
phosphinothricin-based herbicide,
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ividuals were planted in multipots
carry the transgene construct. To
BC_4 plants carrying the transgene
th the *pat* and *Bt* genes. Resistant
eaf-sampled for molecular marker

analyses. Results of marker analyses were made available at the latest two weeks after flowering. A single plant was selected, of which all backcross-derived embryos were rescued and transferred onto tissue culture medium. Plantlets that developed from these embryos first underwent a greenhouse acclimation phase, while still growing on tissue culture medium, before being transplanted into multipots. Backcross cycles lasted, on average, four months.

Molecular marker analyses

Restriction Fragment Length Polymorphisms (RFLP's) were used to establish genotypes in all four generations. RFLP detection involved either radioactive or chemiluminescent techniques. For the BC_1 generation, 61 marker-enzyme combinations were chosen from among those revealing polymorphism between donor and recipient. They provided coverage of the entire genome, defining intervals of about 25 cM in size, and contained two loci tightly linked to the *Bt* locus, CG320 and CG415, respectively 5 and 16 recombination units away (Figure 1). For subsequent generations, markers analyzed in the BC_{n+1} generation comprised both those for which the selected BC_n plant was heterozygous, or tightly linked ones, and additional ones located in chromosomal segments for which the selected BC_n plant was heterozygous (Table 1). Marker map positions were obtained from independent reference populations and confirmed by analysis of segregation in the BC_1 generation.

Selection procedure

At each generation plants were ranked based both on the percentage of homozygous recurrent-parent-genotype and on the extent of linkage drag around the *Bt* locus, in an attempt to integrate both criteria. Plants for which two or more adjacent markers had missing values were not included in the analyses. Success or failure of the pollinations also contributed to the selection procedure. One single plant was selected at each generation: the best ranking one of those for which a backcross progeny of size 100 or more (50 or more for the BC_3 selection) was available.

Results and discussion

Selection for the gene of interest

The observed segregation ratios for phosphinothricin resistance (Table 1) were not significantly different ($P < 0.05$) from the expected 1:1, as shown by Chi-square tests.

Recurrent parent genotype recovery

Statistics for the genotyped plants are summarized in Table 1. Calculations were performed taking the whole genome into account, including the *Bt* locus. The "perfect" backcross-derived plant therefore counts one heterozygous chromosome segment, that

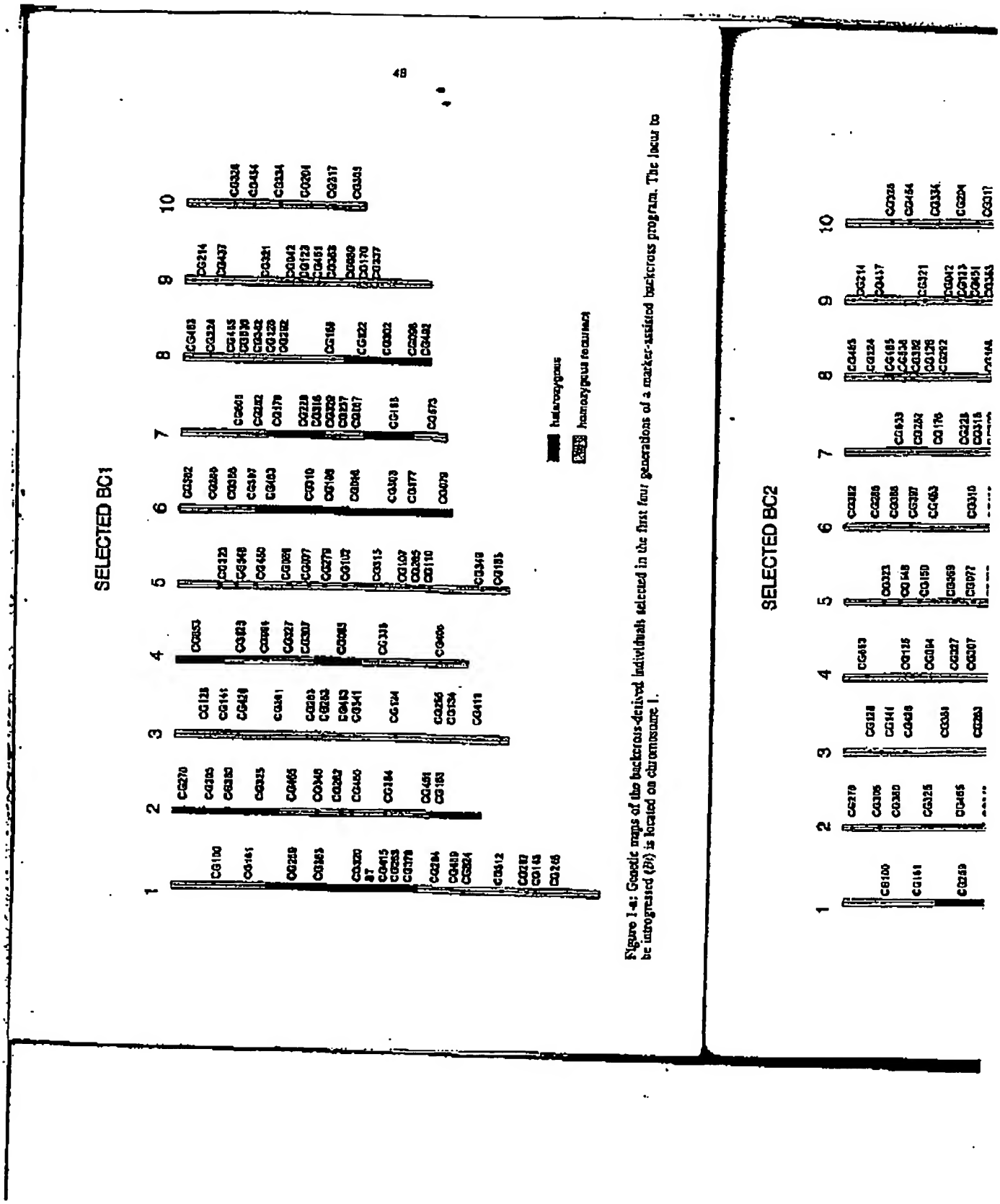

Figure 1-4: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

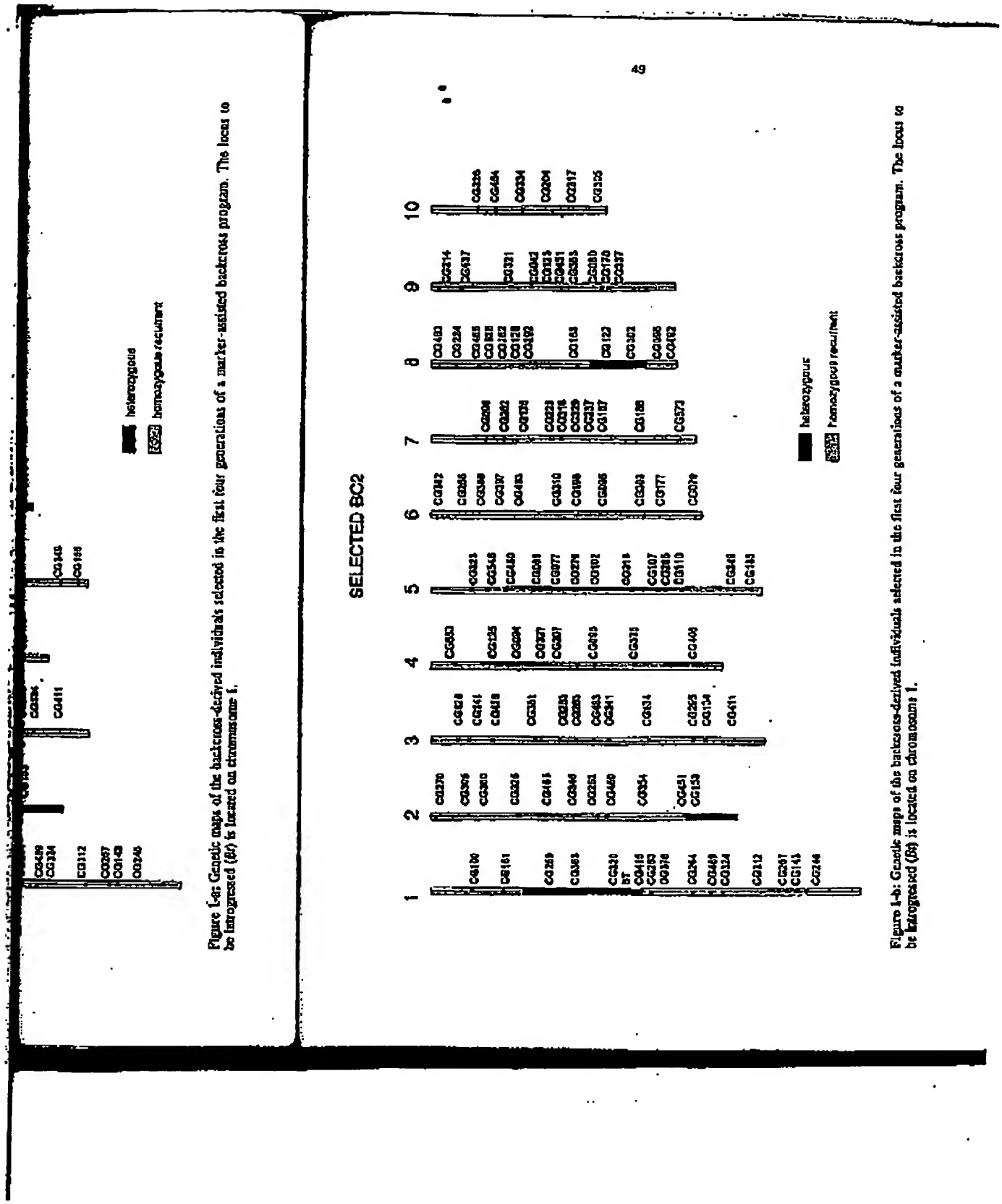

Figure 1-a: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (fw) is located on chromosome 1.

Figure 1-b: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (fw) is located on chromosome 1.

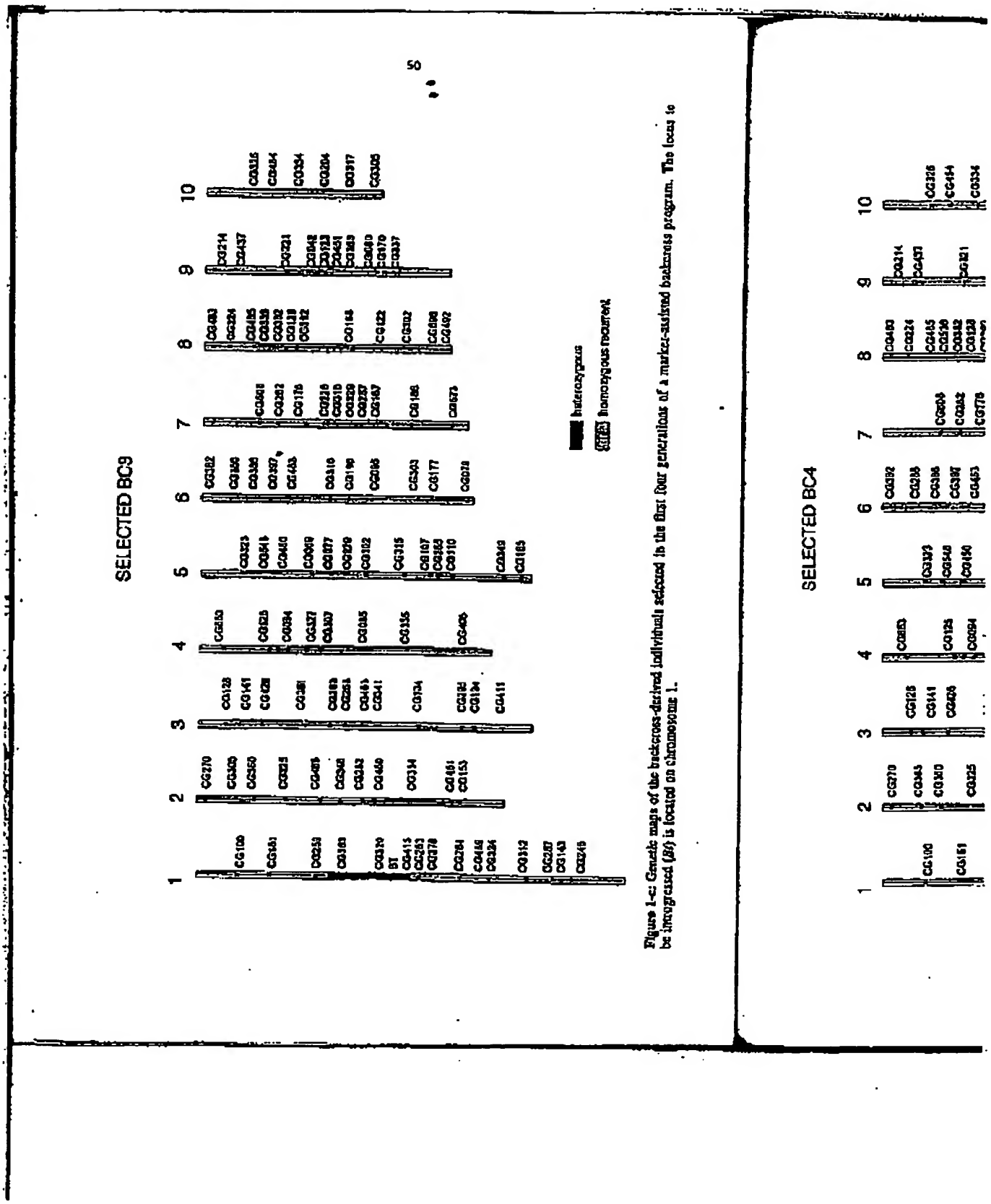

Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (*Bt*) is located on chromosome 1.

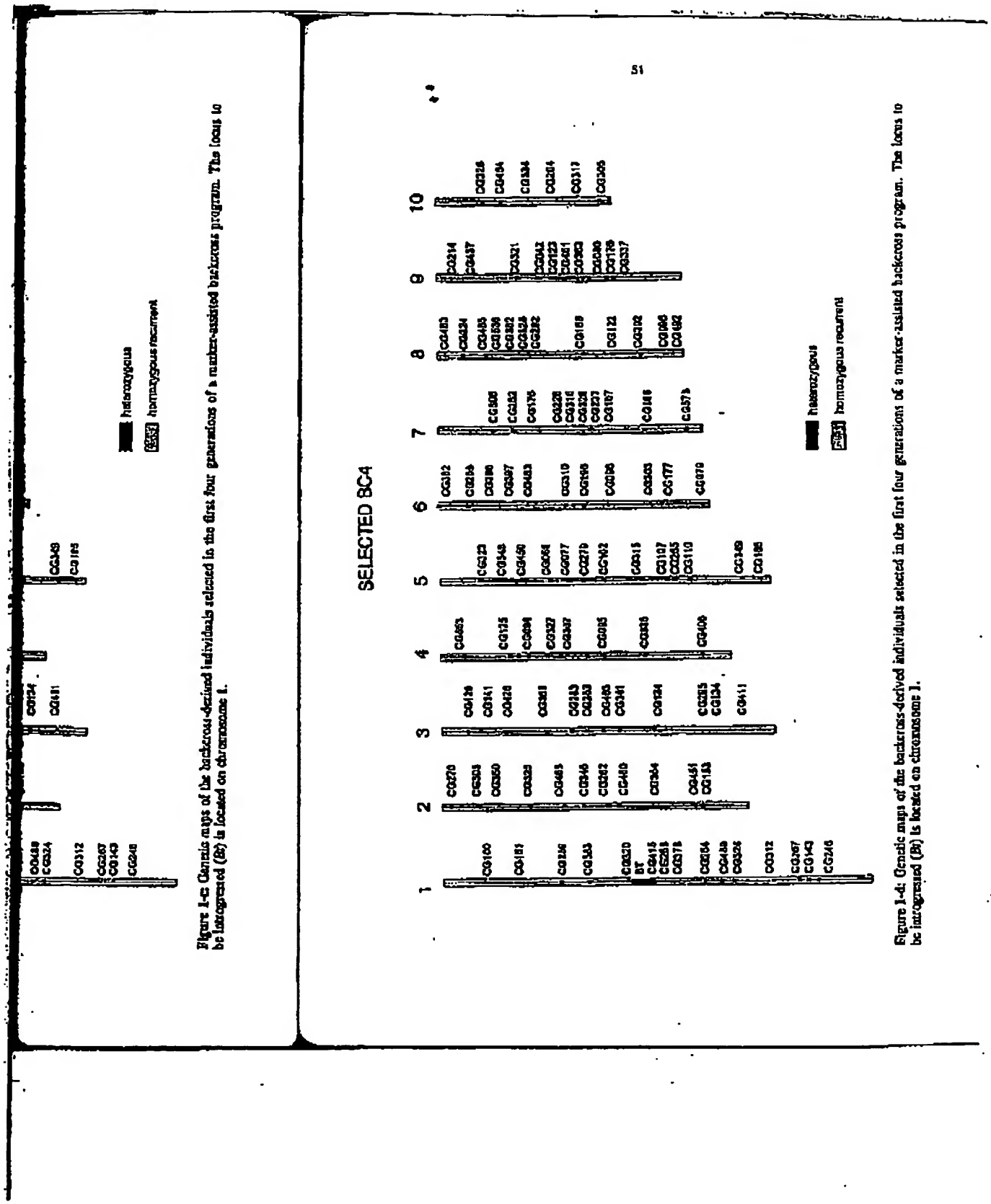

Figure 1-c: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Bt) is located on chromosome 1.

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Figure 1-d: Genetic maps of the backcross-derived individuals selected in the first four generations of a marker-assisted backcross program. The locus to be introgressed (Bt) is located on chromosome 1.

Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% chromosome	RFLP monitored	nb plants	% homozygous recurrent	nb heterozygous

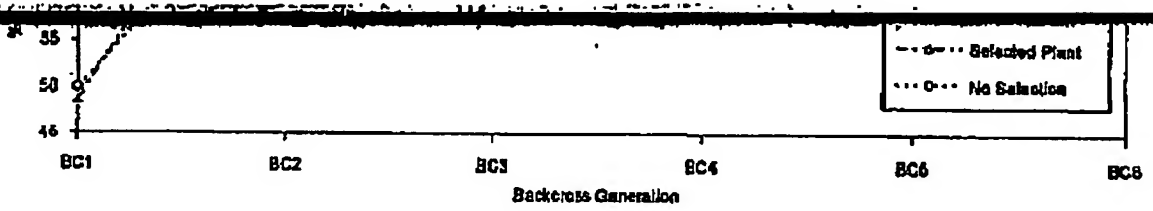

Figure 2: Recovery of recurrent parent genotype through backcrossing, with or without marker-assisted selection

Table 1: Proportion and characteristics of plants carrying the genes of interest, in the first four generations of a marker-assisted backcross program.

generation	% phosphinothricin resistant plants	RFLP genotyping			nb plants analyzed *	% homozygous recurrent parent genotype				nb heterozygous chromosome segments ***			
		nb plants	nb loci	nb datapoints		mean	std dev	5-best mean **	selected plant	mean	std dev	5-best mean **	selected plant
BC1	49.05	96	61	5856	87	48.72	10.35	68.31	70.45	11.91	2.17	7.75	6
BC2	44.66	51	22	1342	30	83.42	5.64	91.95	90.84	3.03	1.54	3.20	3
BC3	48.32	72	10	720	71	93.63	1.85	96.82	98.03	2.20	0.71	1.80	1
BC4	-	26	3	78	26	98.23	0.49	99.09	99.38	1.00	0.00	1.00	1

* Plants for which two or more adjacent markers had missing values were not included in the analyses
** Mean value of the five individuals having the five highest percentages of homozygous recurrent parent genotype.
*** including the segment carrying the transgene construct.

comprising the *Bt* locus. It also displays 99.36% of homozygous recurrent-parent-genotype. The remaining 0.64% corresponds to the average relative length of the chromosome segment containing the *Bt* locus, which depends on the two flanking markers chosen.

The mean percentage of homozygous recurrent-parent-genotype of the BC_1 generation was slightly lower than the expected 50%. This can be explained by linkage drag around the *Bt* locus, given that this percentage was computed based only on plants selected for heterozygosity at the *Bt* locus. For all other backcross generations the mean percentage of homozygous recurrent-parent-genotype was much higher than what would have been observed, should no selection have been done (Figure 2).

The percentage of homozygous recurrent-parent-genotype of the selected plant (Table 1) and the average of the five largest values (Table 1) were always very similar to one another, and much superior to the population mean value (Figure 2). The percentage of homozygous recurrent-parent-genotype of the selected plant was found only once, in the BC_2 generation, to be smaller than the average of the five largest values. This corresponded to the only time when the selected plant was not the one with the maximum percentage of homozygous recurrent-parent-genotype. The plant had been selected because it displayed a favorable recombination on one side of the *Bt* locus (Figure 1).

The percentage of homozygous recurrent-parent-genotype of the selected BC_1 plant was almost equal to that of an unselected BC_2, that of the selected BC_2 was larger than that of an unselected BC_3, that of the selected BC_3 was barely smaller than that of an unselected BC_6, and that of the selected BC_4 was equal to that of the "perfect" backcross-derived plant, given the set of markers that was used. Such rates of recurrent parent genotype recovery are consistent with results of simulation analyses. Tarboe *et al.* (1994) who used the maize genome as a model reported that three backcross generations and 80 markers were needed to recover 99% of recurrent parent genotype.

Number of donor chromosome segments

The number of heterozygous chromosomal segments decreased from one backcross generation to the next. Plants selected at each generation were not necessarily those which had the lowest number of heterozygous chromosomal segments (Table 1). However, with the set of markers used, BC_3 and BC_4 plants were recovered which contained only one heterozygous chromosomal segment: that comprising the *Bt* locus.

Linkage drag

Linkage drag around the *Bt* locus was estimated, relative to the length of chromosome 1. Its value was found to lie between 24.0 and 48.4% for the selected BC_1 individual, between 17.6 and 34.8% for the selected BC_2, between 2.0 and 24.0% for the selected BC_3, and between 0.0 and 8.4% (respectively 0.0 and 14.5 cM) for the selected BC_4.

The two values given for each g
correspond to extreme positions o
flanking the transgene construct locu
BC_4 is likely to be less than 1.3%
appear to be somewhat high, reflec
drag, it is much lower than what
(Stam and Zeven 1981; Tanksley *et*
of tomato cultivars obtained by a l
Tanksley (1989) found that the sizes
cM.

Conclusion

These results clearly demonstr
quality advantages over classical
through backcrossing. Only four ba
than a year and a half from plan
genotypically fully converted. Neve
genotype could proceed even faste
appropriate protocol and resources
allocated.

Comparison of BC_6-derived
markers and agronomic performanc
order to confirm the completeness o

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14.5 cM) for the selected BC_4.

The two values given for each generation are extreme values of linkage drag, which correspond to extreme positions of the crossing-overs in the marker-defined intervals flanking the transgene construct locus. Therefore the true linkage drag value of the selected BC_4 is likely to be less than 1.3% of the genome. Although this maximum value may appear to be somewhat high, reflecting the limited selection pressure put here on linkage drag, it is much lower than what would be expected from classical backcross programs (Stam and Zeven 1981; Tanksley *et al.* 1989). Practically, in a study of *Tm-2* conversions of tomato cultivars obtained by a large number of classical backcross cycles, Young and Tanksley (1989) found that the sizes of the introgressed fragments ranged between 4 and 51 cM.

Conclusion

These results clearly demonstrate that molecular markers provide important time and quality advantages over classical procedures for the production of near-isogenic lines through backcrossing. Only four backcross generations were necessary to recover, in less than a year and a half from planting of the BC_1's, individuals which appeared to be genotypically fully converted. Nevertheless, it is likely that recovery of recurrent parent genotype could proceed even faster than in the experiment described herein, should the appropriate protocol and resources (population size, number and position of markers) be allocated.

Comparison of BC_4-derived lines with the recurrent parent for both morphological markers and agronomic performance (including hybrid performance) will be performed in order to confirm the completeness of the conversion.

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C

Marker-assisted Selection in Backcross Breeding

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Abstract. The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Genetic markers can increase the effectiveness of backcrossing by 1) increasing the probability of obtaining a suitable conversion, and 2) decreasing the time required to achieve an acceptable recovery. Simulation and field results indicated that, for a genome consisting of ten 200-cM chromosomes, basing selection on 40 or 80 markers in 50 BC individuals that carry the allele being transferred can reduce the number of backcross generations needed from about seven to three.

The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Usually, the trait being transferred is controlled by a single gene, but highly heritable traits that are more complexly inherited have also been transferred successfully by backcrossing; for example, maturity in maize (Klein and Sauer, 1961; Stuber, 1976). Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm.

Several plant breeding textbooks give good descriptions of the backcross procedure (Allard, 1960; Fehr, 1987). A donor parent (DP) carrying a trait of interest is crossed to the recurrent parent (RP), an elite line that is lacking the trait. The F_1 is crossed back to the RP to produce the BC_1 generation. In the BC_1 and subsequent backcross generations, selected individuals carrying the gene being transferred are backcrossed to the RP. The expected proportion of DP genome is reduced by half with each generation of backcrossing. Ignoring effects of linkage to the selected DP allele being transferred, the percentage recurrent parent (%RP) genome expected in each backcross generation is calculated as:

$$\%RP = 100\,[1 - (0.5)^{n+1}]$$

where n is the number of backcrosses.

Backcrossing of selected plants to the RP can be repeated each cycle until a line is obtained that is essentially a version of the RP that includes the introgressed allele. After six backcrosses, the expected recovery is >99% (Table 1).

Until recently, discussions of the recovery of the RP genome during backcrossing have emphasized the expected values for %RP shown in Table 1, and have largely ignored the genetic variation for %RP that exists around the expected mean. With the development of genetic markers capable of providing good genome coverage, there has been interest in taking advantage of that variation to increase the efficiency of backcrossing.

Selection for RP marker alleles can increase greatly the effectiveness of backcross programs by allowing the breeder to 1) select backcross plants that have a higher proportion of RP genome, and 2) select backcross individuals that are better conversions near a mapped donor allele being transferred (i.e., select for less linkage drag). Expressed in practical terms, using genetic markers to assist backcrossing can 1) increase the probability of obtaining a suitable conversion, and 2) decrease the time required to achieve an acceptable recovery.

Issues to consider when planning a marker-assisted backcross program include 1) the time advantage of using markers to assist backcrossing, 2) the number of markers needed, and 3) the number of genotypes to evaluate. In this report, we use results from previous literature, computer simulation, and empirical studies to provide some guidelines.

Table 1. *Expected recovery of recurrent parent (RP) genome during backcrossing, assuming no linkage to the gene being transferred.*

Generation	%RP
F_1	50.0000
BC_1	75.0000
BC_2	87.5000
BC_3	93.7500
BC_4	96.8750
BC_5	98.4375
BC_6	99.2188
BC_7	99.6094

[1]Formerly with Purdue University, West Lafayette, Ind.

Appendix 2

Materials and methods

The maize genome was the model for the simulation. The simulated genome consisted of 200-cM chromosomes. Simulation of crossing over was based on a Poisson distribution with a mean of 2.0 ($\lambda = 2$) (Hanson, 1959), which, on average, generated one cross over for every 100-cM length. The simulations reported here assume no interference. Codominant genetic markers were evenly distributed in the genome and sites of the donor gene were randomly assigned to genome locations.

Simulations were conducted with the following parameters:

Number of progeny: 100 or 500.
Backcross generations: BC_1, BC_2, and BC_3.
Number of markers: 20, 40, 80, or 100.
Number selected to form the next BC generation: 1 or 5.

Selection was based on 1) presence of the donor allele and 2) high %RP). %RP was calculated as the average of the (one or five) selected individuals. Values presented are the mean of 50 simulations.

Results

In the computer simulation study, all methods modeled greatly increased the speed of recovering the RP genome compared to the expected recovery with no marker-assisted selection (compare Tables 1 and 2). At least 80 markers were required to recover 99% of the RP genome in just three BC generations (Table 2). Use of at least 80 markers and 500 progeny allowed recovery of 98% RP in just two BC generations. Response to selection was diminished only slightly by spreading the effort over five selections. Using markers, the number of backcross generations needed to convert an inbred is reduced from about seven to three.

By the BC_2 generation, there appears to be no practical advantage to using 500 vs. 100 individuals. If the presence of the donor trait in the backcross individuals can be ascertained before markers are genotyped, then only half the number of individuals indicated in the tables will need to be analyzed.

When a small number of markers are used, they quickly become non-informative; i.e., selection causes the marker loci to become fixed for the RP type before the rest of the genome is fully converted (Table 3; Hospital et al., 1992). This situation was most prominent in the larger populations, where a higher selection intensity placed more selection pressure upon the marker loci. Accordingly, it is of interest to consider how closely the estimation of %RP based on markers reflects the actual genome composition. The combination of estimation of %RP based on fewer markers and subsequent selection tends to bias the estimates upward (compare Tables 2 and 3).

The results from the simulation compare well with real field data. In a typical example, 50 BC_1 plants carrying the gene being transferred were genotyped at 83 polymorphic RFLP loci (note that this corresponds to a population size of 100 unselected plants in Tables 2 and 3). The five best BC_1 recoveries had estimated %RP values of 85.9%, 82.7%, 82.0%, 81.4%, and 81.2%. After evaluating 10 BC_2 plants from each selected BC_1, the best BC_2 recovery had an estimated %RP of 94.6%.

Discussion

The simulations (Table 2; Hospital et al., 1992) and our experience indicate that four markers per 200-cM chromosome is adequate to greatly increase the effectiveness of selection in the BC_1. However, using only four markers per 200 cM will likely make it very difficult to map the location of the gene of interest. Adequate summarization of the data is an important

Table 2. *Percent recurrent parent genome during marker-assisted backcrossing.*

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_1	84.5	84.5	84.2	84.0	89.9	90.7	90.2	90.5
BC_2	93.0	93.2	95.8	97.2	96.5	97.7	98.5	98.6
BC_3	97.4	97.6	98.9	99.2	97.7	98.3	99.4	99.5
				Five selected				
BC_1	82.9	83.1	84.9	84.7	87.7	88.1	88.9	84.9
BC_2	93.7	95.0	95.8	95.7	95.5	96.8	97.8	97.9
BC_3	97.1	98.3	98.6	98.9	97.3	98.5	99.3	99.3

Table 3. *Estimates of percent recurrent parent genome, based on marker loci.*

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_2	98.7	97.8	95.6	97.1	100.0	99.1	98.6	98.0
BC_3	100.0	99.8	99.3	99.5	100.0	100.0	99.9	99.2
				Five selected				
BC_2	96.4	96.5	96.2	95.8	100.0	98.5	98.9	98.2
BC_3	99.9	99.8	99.5	99.1	100.0	100.0	99.9	99.8

gen of a marker-assisted backcross program. Ideally, the marker used can supply data that can be represented as alleles of loci with known map position. Estimation of %RP, mapping the position of the locus of interest, and graphical display of the result (Young and Tanksley, 1989) are all useful in understanding and controlling the specific backcross experiment being conducted.

It appears that, with the use of genetic markers, the portion of the RP genome that is not linked to the allele being transferred can be recovered quickly and with confidence. The recovery of RP will be slower on the chromosome carrying the gene of interest. A considerable amount of linkage drag is expected to accompany selection for the DP allele in a backcross program. For a locus located in the middle of a 200-cM chromosome, the length of the DP chromosome segment accompanying selection is expected to be 126, 63, and 21 cM in the BC_1, BC_2, and BC_3 generations, respectively (Haaren, 1939; Navebra and Barbedillo, 1992). Our observations support the recommendation of Hospital et al. (1992) that preference be given to the selection for recombinants proximal to the allele of interest, but that selection for recovery of the RP elsewhere in the genome also be considered. This two-stage selection can probably be done quite effectively ad hoc by the breeder once the data is adequately summarized; however, Hospital et al. suggest ways to incorporate the two criteria into a selection index such that each component of selection is assured appropriate weighting.

Use of genetic markers can greatly increase the effectiveness of backcrossing, and they should be used in any serious backcrossing program if resources are available to the breeder.

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MaizeGDB
Maize Genetics and Genomics Database

bnlg1014 (locus)

This locus is also known by the following names:
bmc1014
bng1014

Type: Probed Site
Species: Zea mays ssp. mays
Linkage Group: 1
Arm: S (short arm)

Map Coordinates: (* indicates the locus is on the backbone)

Map	Coordinate	Bin
A632/rtcs1 1999	20.00	1.01
bins 1	1.01	1.01
BNL 2002 1	41.68	1.01
Chromatin IBM 2003 1 *	82.80	1.01
IBM IDP +MMP bd (ver 4) 1	48.91	1.01
IBM neighbors v.2 1 *	76.40	1.01
IBM1 1 *	76.40	1.01
IBM2 1 *	82.80	1.01
IBM2 2004 neighbors 1 *	82.80	1.01
IBM2 2004 neighbors frame 1 *	82.80	1.01
IBM2 FPC0402 genetic neighbors 1 *	83.02	1.01
IBM2 neighbors 1 *	82.60	1.01
IBM2 neighbors frame 1 *	82.80	1.01
LHRF Gnp2004 1 *	16.00	
Pioneer composite 1999 1	20.70	1.01
SSR Consensus 1	24.50	1.01
SSR IBM 1 *	66.10	1.01
SSR Tx303xCO159 2002 1 *	21.90	1.01
SSR Tx303xCO159 2003 1 *	22.00	1.01

SSRs
p-bnlg1014 (via SSR PCR)

Primers and Enzymes:

Primer/Enzyme	Probe
CACGCTGTTTCAGACAGGAA	p-bnlg1014
CGCCTGTGATTGCACTACAC	p-bnlg1014

Anchored BACs: (BACs identified to be anchored by probes for this locus):

b0074A07	b0092D02	b0138L14	b0008N23
b0036L14	b0182A10	b0284B14	